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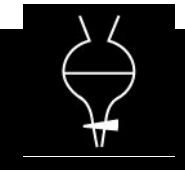
RESTRICTED-ACCESS MEDIA: SOLID-PHASE EXTRACTION



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Introduction

For the determination of drugs and their metabolites in serum or plasma by high performance liquid chromatography (HPLC), tedious and time-consuming pretreatment procedures such as liquid–liquid extraction, solid-phase extraction (SPE) or membrane-based extraction are often required. Among those pretreatment procedures, SPE is the most widely used for extraction of target compounds in biological fluids. However, direct injection of serum or plasma samples onto HPLC or SPE materials causes protein denaturation with accumulation of materials on the sorbent, resulting in undesired loss in the capacity and selectivity of the sorbent. Thus, it is essential to remove serum or plasma proteins before loading the samples onto the HPLC or SPE sorbents. Recently, restricted access media (RAM) materials were introduced for direct injection of proteinaceous samples onto the HPLC or SPE materials. With RAM materials large molecules such as proteins are eluted in the void volume without destructive accumulation because of restricted access to some surfaces, while allowing small molecules such as drugs and their metabolities to reach the hydrophobic, ion-exchange or affinity sites and be separated. One approach uses an internal-surface reversed-phase (ISRP) material, produced from porous silica gels, which has hydrophobic interior and hydrophilic exterior surfaces, as shown in **Figure 1**. The ISRP–GFF material (GFF = glycine-L-phenylalanine-L-phenylalanine) was prepared from covalently modified glyceryl-propyl (i.e. diol) phases by attachment of the tripeptide GFF, bonded via the amino groups to the glycerylpropyl groups. The phenylalanine moieties were then cleaved from the external surface of the silica with carboxypeptidase A, which is too large to enter the small pores. After this enzymatic treatment, the glycerylpropyl moieties and glycine residues remain on the external surface. Because the ISRP concept was innovative for drug determinations in serum, many RAM materials were subsequently developed. Another RAM material based on silica gels is shielded hydrophobic phase (SHP), which consists of a

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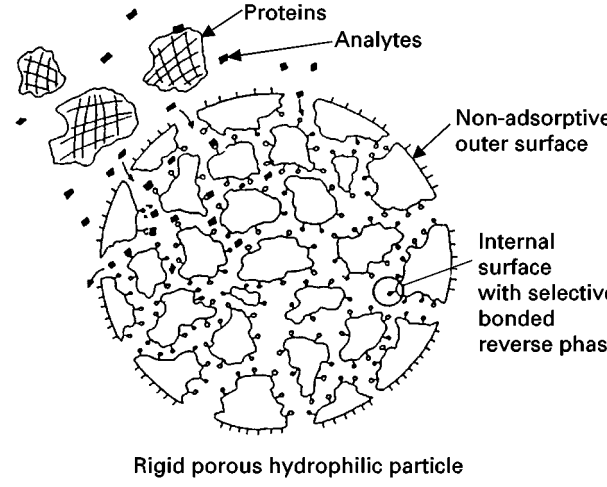

Figure 1 Schematic representation of an internal-surface reversed-phase (ISRP) material. Proteins do not adsorb on the hydrophilic exterior surfaces and do not penetrate into the hydrophobic interior surfaces, while analytes can reach the interior surfaces and be separated. (Reproduced with permission from Perry JA (1991) The internal surface reversed phase. Concept and applications. *Journal of Liquid Chromatography* 13: 1047–1074.)

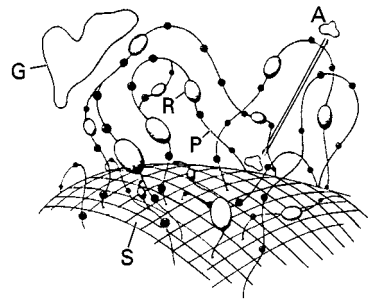

Figure 2 Schematic representation of a shielded hydrophobic phase (SHP) material. S = silica gel matrix; R = hydrophobic pocket; P = hydrophilic network; G = large unretained protein; A = small retained analyte. (Reproduced with permission from Gisch DJ, Hunter BT and Feibush B (1988) Shielded hydrophobic phase: a new concept for direct injection analysis of biological fluids by high-performance liquid chromatography. *Journal of Chromatography* 433: 264–268.)

hydrophilic polyoxyethylene network embedded with phenyl groups, bonded to both the external and internal surfaces of the particles (**Figure 2**). Other RAM materials based on silica gels include semipermeable surface, dual zone and mixed function phase materials.

RAM materials based on polymer beads have also been developed. For example, one polymer-based RAM material was prepared from porous uniformly sized poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads. Hydrolysis of the epoxide groups to diols can be carried out exclusively within the large pores of the medium through the use of a polymeric catalyst, polystyrenesulfonic acid (average molecular weight, 141 000 Da). The epoxide groups remaining in the small pores after hydrophilization of the large pores were then reacted with either hydrophobic C_{18} or phenyl groups, or more polar diethylamino groups. The pore-size selective modification of porous materials provided the RAM materials as shown in **Figure 3**.

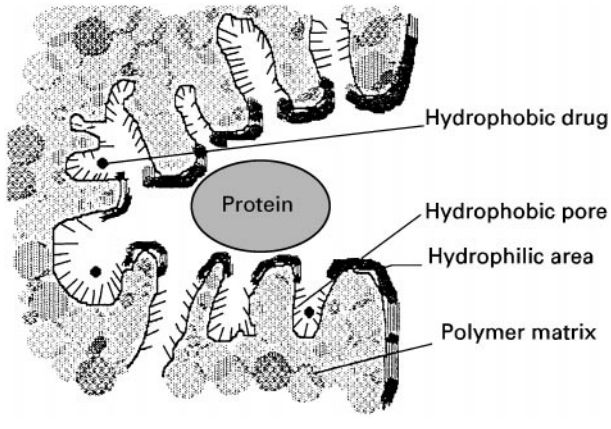

Figure 3 Schematic representation of a polymer-based RAM material modified in pore-size selective fashion using a polymer catalyst. (Reproduced with permission from Smigol V, Svec F and Fréchet JMJ (1994) Novel uniformly sized polymeric stationary phase with hydrophilized large pores for direct injection HPLC determination of drugs in biological fluids. *Journal of Liquid Chromatography* 17: 891–911.)

RAM materials could be used for direct serum injection assays of drugs as HPLC or SPE materials. The former materials have been designed and used preferentially as packings for large-size (150 × 4.6 mm i.d.), i.e. analytical columns. In this case the extraction and separation of analytes take place simultaneously. For SPE RAM, sorbents were packed into a small (typically 5–30 mm × 3–4.6 mm i.d.) precolumns connected to an analytical column via a six-port valve, i.e. switching valve. In the coupled-column mode this is a sequential process. The two approaches for the extraction and analysis of the target compounds in biological fluids by HPLC are compared in **Table 1**.

Single-column Mode

When using the RAM materials, the ionic strength and pH of an eluent, and the content of organic modifier are limited in order to prevent precipitation of serum proteins. For the ISRP–GFF materials, the recommended eluent pH range was 6.0–7.5. The recovery of serum proteins was low at acidic pH. This is due to the electrostatic attractions of the serum proteins having a net positive charge (isoelectric point, p*I* of serum albumin, 4.7) and the external glycine residues having a negative charge. Taking into account the pK_a values of the bound glycine (between 2.3 and 3.0), the recovery of serum proteins might be higher with an eluent pH below 2. However, chemically bonded columns cannot be used for long periods at this pH because of the hydrolysis of the bonded phase. However, for RAM materials such as SHP, whose external surface has no charges, there is no eluent pH limitation. These materials can be used at any pH suitable for siloxane-bonded silicas (pH 2–8). These results demonstrated that external uncharged surfaces should be suitable for the external layers of RAM materials. With regard to the eluent, an ionic strength of 0.05–0.2 was used. The preferred organic modifiers are acetonitrile, 2-propanol, tetrahydrofuran and methanol because they can afford a wide selectivity in controlling solute retention on the accessible hydrophobic surfaces. The content of the organic modifier should be $< 20\%$.

Direct serum injection assays of drugs were carried out on the ISRP–GFF materials. The chromatograms of plasma spiked with probenecid or lidocaine at clinical levels (50 μg mL^{-1} for probenecid, 5.94 μg mL^{-1} for lidocaine) are shown in **Figures 4** and **5**, respectively, together with those for methanolic solutions of the

Table 1 On line sample extraction and analysis: comparison of single-column and coupled-column modes. (Reproduced with permission from Boos K-S and Grim C-H (1999) High-performance liquid chromatography integrated solid-phase extraction in bioanalysis using restricted access precolumn packings. *Trends in Analytical Chemistry* 18: 175–180)

Parameter	*Single-column mode*	*Coupled-column mode*
Matrix elimination and analyte separation	Simultaneous	Sequential
Peak capacity	Low	High
Selectivity	Low	High
Incidence of interferences	High	Low
Sample volume	<100 μL	$\gg 100$ μL
Analyte enrichment	No	Yes
Limit of quantification	Increased	Decreased
Mobile-phase composition	Restricted (pH, additives)	Variable
Detection	UV $>$ 240 nm Fluorescence detection – yes Electrochemical detection – no	No limitation
Column lifetime	Short	Long
Cost/analysis	High	Low

same concentration. The recovery was calculated from the peak–area ratio of a given concentration of the drug dissolved in plasma and methanol. Despite the differences in the bound fractions (83–94% for probenecid and 65–77% for lidocaine), both drugs were almost completely recovered from plasma samples. The large difference in the intensities of the background peaks in these chromatograms is due to the difference in the detection wavelengths. Naturally, the shorter wavelength (220 nm for lidocaine) reveals more matrix peaks at a higher intensity than the longer wavelength (254 nm for probenecid). In

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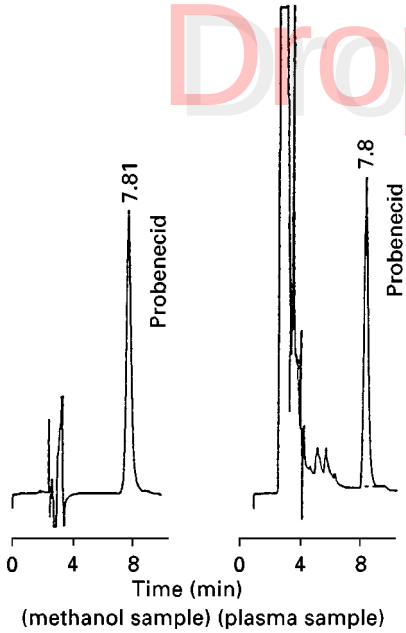

Figure 4 Separation of probenecid from human plasma. Mobile phase, 0.1 M potassium phosphate buffer–tetrahydrofuran (95 : 5), pH 7.0; flow rate, 1.0 mL min^{-1}; stationary phase, ISRP–GFF column, 150 mm × 4.6 mm i.d.; detection, UV (254 nm); injection volume, 10 μL. (Reproduced with permission from Nakagawa T, Shibukawa A, Shimono N *et al.* (1987) Retention properties of internal-surface reversed-phase silica packing and recovery of drugs from human plasma. *Journal of Chromatography* 420: 297–311.)

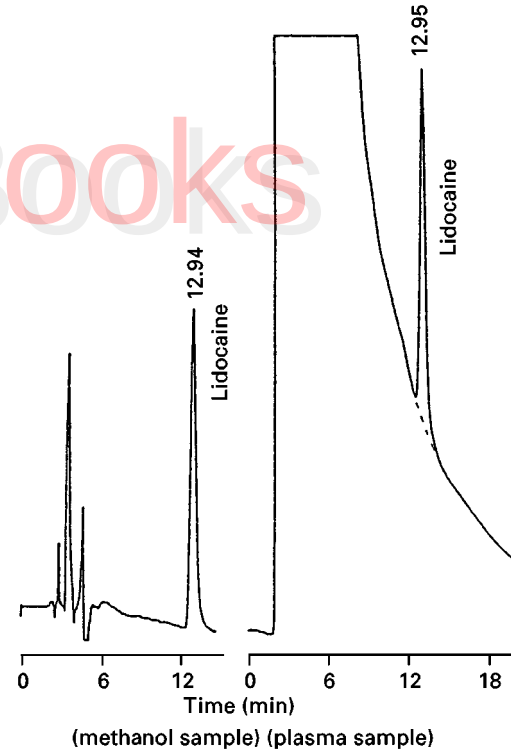

Figure 5 Separation of lidocaine from human plasma. Mobile phase, 0.1 M potassium phosphate buffer–tetrahydrofuran (9 : 1), pH 7.2; flow rate, 0.8 mL min^{-1}; stationary phase, ISRP–GFF column, 150 mm × 4.6 mm i.d.; detection, UV (220 nm); injection volume, 10 μL. (Reproduced with permission from Nakagawa T, Shibukawa A, Shimono N *et al.* (1987) Retention properties of internal-surface reversed-phase silica packing and recovery of drugs from human plasma. *Journal of Chromatography* 420: 297–311.)

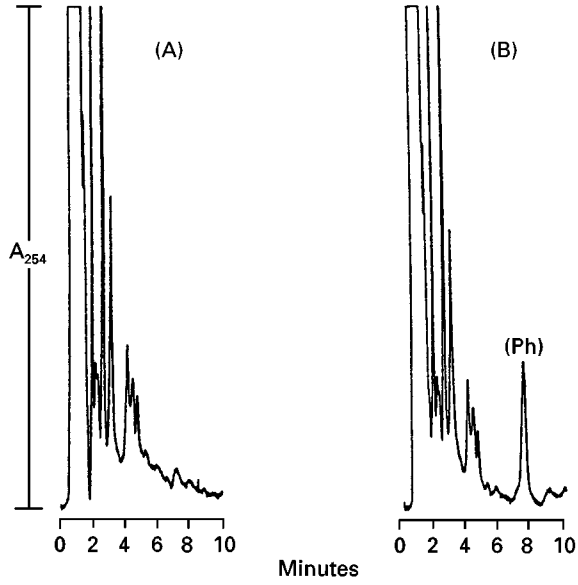

Figure 6 Chromatograms of fetal bovine serum (A) and phenytoin (Ph)-spiked fetal bovine serum (B) at pH 2.5. Chromatographic conditions: column, SHP column (150 mm × 4.6 mm i.d.); mobile phase, acetonitrile – 50 mM KH_2PO_4 (pH 2.5) (15 : 85); flow rate, 2.0 mL min^{-1}; temperature, ambient; detection, UV at 254 nm, 0.008 aufs; injection volume, 25 μL. (Reproduced with permission from Gisch DJ, Feibush B, Hunter BT *et al.* (1989) A new HPLC concept for direct analysis of drugs in biological matrices: shielded hydrophobic phase. *BioChromatography* 4: 206–215.)

both cases the eluent pH was about 7. However, phenytoin was not eluted under the neutral conditions on the SHP materials, but when the eluent pH was adjusted to 2.5, it was eluted and resolved from serum matrix components (**Figure 6**). Because of the presence of secondary amines on phenytoin, the retention factor of phenytoin was decreased by reducing the eluent pH. Since the SHP materials had no charged groups on the external surface as described above, they could be used at eluent pH 2.5.

In the above applications, less than 100 μL of serum sample was injected. At higher sample volumes, analyte peaks were broadened and a plateau peak was observed. The plateau peak is dependent on the unbound fraction of analyte. Whether the broadened or plateau peak appears; that is, when the unbound drug fraction is higher, we can inject a larger sample volume for direct serum injection assays of the drug without peak-broadening.

On the other hand, both free and total drug concentrations could be simultaneously determined by injecting such a larger sample volume that the plateau peak of a drug appears. **Figure 7** shows the chromatogram of 8.00 μg mL^{-1} carbamazepin (CBZ) in human plasma. CBZ was well separated from the blank peak and gave a clear and wide plateau. The CBZ concentration calculated from this plateau height was 1.97 μg mL^{-1}, which agreed with the free CBZ concentration determined by means of ultrafiltration (2.08 μg mL^{-1}). Furthermore, it is interesting that the CBZ concentration calculated from the area of this plateau was 8.06 μg mL^{-1}, in agreement with the total CBZ concentration of this plasma sample. This implies that both free and total drug concentrations can be determined simultaneously by a single analysis based on the height and area of the drug plateau, respectively. However, it is required to inject a large sample volume (in this case, 1.8 mL plasma sample) in order to observe the plateau peak. Further, the plateau peak cannot always be separated from blank peak, dependent on the drug separated.

Coupled-column Mode

As shown in **Table 1**, the advantages of a coupled-column mode include separation selectivity (ability to couple precolumns and analytical columns of different selectivity), detection sensitivity (analyte enrichment due to larger sample volumes and reduced number of interfering peaks), and higher variability of mobile phases and detection modes. In recent times RAM materials have mainly been used in the coupled-column mode.

Figure 8 shows a representative chromatogram obtained after the direct injection of an untreated human serum sample onto a RAM precolumn. The injection was followed by fully automated online extraction and subsequent separation of antiepileptic

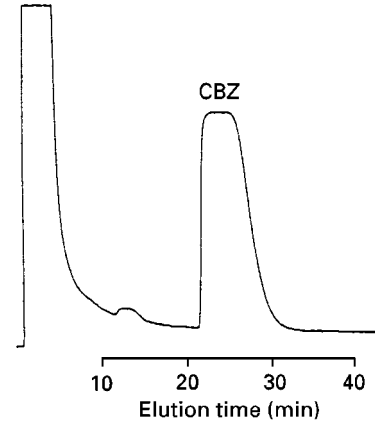

Figure 7 Determination of unbound and bound concentrations of carbamazepine (CBZ) in human plasma. Total concentration of CBZ is 8 μg mL^{-1}. Stationary phase: ISRP–GFF column (150 mm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, $I = 0.17$). Flow rate: 1.2 mL min^{-1}. Detection: UV 300 nm. Column temperature: 37°C. Injection volume: 1.8 mL. (Reproduced with permission from Shibukawa A, Nakagawa T, Nishimura N *et al.* (1989) Determination of free drug in protein binding equilibrium by high-performance frontal analysis using internal-surface reversed-phase silica support. *Chemical & Pharmaceutical Bulletin* 37: 702–706.)

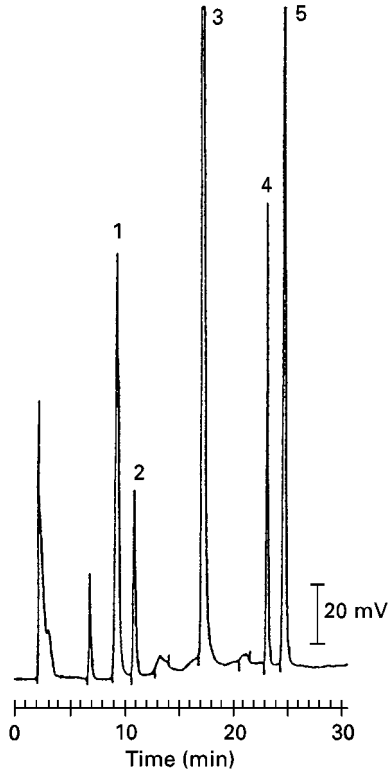

Figure 8 Coupled-column analysis of antiepileptic drugs in serum. Precolumn: 25 mm × 4 mm i.d., LiChrospher RP-18 ADS (particle size, 25 μm); analytical column: 250 mm × 4 mm i.d., LiChrospher 60 RP-Select B (particle size, 5 μm); loading mobile phase: 0.5 M monobasic sodium phosphate (pH 4.0) for 0 min at 0.5 mL min^{-1}; transfer mobile phase: 5 : 95 (v/v) acetonitrile–water for 5 min at 0.5 mL min^{-1}; separation mobile phase: acetonitrile–water with a linear acetonitrile gradient from 5% to 34% in 34 min at 0.5 mL min^{-1}; detection: UV absorbance at 205 nm; sample: 100 μL of analyte-spiked serum. Peaks: 1 = ethosuximide (10.3 nmol), 2 = primidone (1.8 nmol), 3 = phenobarbital (6.6 nmol), 4 = carbamazepine (1.3 nmol), 5 = phenytoin (2.5 nmol). (Reproduced with permission from Boos K-S and Rudolphi A (1997) The use of restricted-access media in HPLC. Part I. Classification and review. *LC–GC* 15: 602–611.)

drugs on a conventional analytical reversed-phase HPLC column. The precolumn (25 mm × 4 mm i.d.) packed with one of ISRP materials (particle size, 25 μm) can tolerate 2000 injections or more of 50 μL of human plasma.

Future Trends

Since the invention of the ISRP–GFF materials, various RAM materials have been developed for direct serum injection assays of drugs with single- and coupled-column modes. However, they are lacking in selectivity because hydrophobic or ion-exchange ligands are used as the analyte binding ligands. In the future, more selective ligands such as immunoaffinity or chemoaffinity ligands or molecularly imprinted polymers have the potential to further improve selectivity and sensitivity in bioanalysis.

Further Reading

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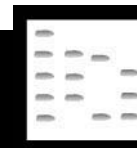
Short Books

REVERSED-FLOW GAS CHROMATOGRAPHY



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Introduction

Gas chromatography (GC) is a technique that is used not only to separate substances from each other, but also to 'separate' physicochemical quantities by